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13. ABSTRACT *(Maximum 200*

Angiogenesis is critical for breast cancer growth and metastasis. This process is controlled by chemical signals known as angiogenic factors. To date, little is known about the source or regulation of angiogenic factor expression. We hypothesize that in human breast cancer, the breast cancer cells are the source of angiogenic factors. Further, this angiogenic factor expression is directly under the control of cytokines and other factors such as fibrin that are normally found in healing wounds. In order to study this question, we are examining the expression and regulation of angiogenic factor expression, specifically IL-8. in human breast tissue, an *in vitro* model of human breast cancer cells, and in an *in vitro* model in immunodeficient mice. We have demonstrated that IL-8 is present in breast cancer specimens by immunohistologic analysis, and in breast cancer homogenates. We have also demonstrated that IL-1 is a potent inducer of IL-8 expression by estrogen independent breast cancer cells. Our results from Years 01 and 02 confirm our original hypothesis, and have set the ground work for the mouse model studies of Years 03 and 04. In the future, treatment of breast cancer may include specific agents designed to interfere with angiogenesis.

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FOREWORD

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Table of Contents

Introduction

Breast cancer is the most common malignancy in women in the United States. The overall survival for all patients with this diagnosis is approximately 70%. Factors associated with poor prognosis include the size of the tumor, lymph node metastases, or invasion of neural and vascular structures. Recently, it has become clear that patients whose tumors demonstrate a high degree of angiogenesis have a shorter disease free survival, and that these patients are more likely to develop distant metastases [1]. It is also well accepted that tumor can not grow larger than several millimeters in diameter (approximately 10^6 cells) without in-growth of additional blood vessels [2]. Angiogenesis has been shown to be critical for the growth and metastasis of human breast cancer [3]. Understanding the underlying mechanisms and factors that control angiogenesis will provide not only insights into the basic mechanisms of tumor growth and metastasis, but also will likely provide novel new therapeutic approaches to invasive breast cancer.

Although the relationship between neovascularization and poor outcome in breast cancer is well established, the mechanism of this relationship is not well understood. Undoubtedly, multiple angiogenic factors (AF) are involved in the recruitment and development of tumor angiogenesis [4]. The possible sources of these factors include the tumor cells themselves, tissue cells or infiltrating leukocytes. Previous studies have demonstrated that AF function by inducing vascular endothelial cell proliferation and/or migration. A variety of AF have been recently identified, and include Interleukin 8 (IL-8), Vascular Endothelial Cell Growth Factor (VEGF) , and basic Fibroblast Growth Factor

(bFGF).

We and others have hypothesized that tumors behave as wounds that do not heal[5, 6]. Normal wounds heal as a result of infiltration of leukocytes and other cells releasing signaling factors which influence the local micro-environment. As a result, a multitude of reactions occur which result in the promotion of wound healing. These reactions include production of fibrin, collagen and other repair factors, as well as an increase in local blood flow to support these activities. This increased blood flow is a result of both local vasodilatation as well as angiogenesis. We hypothesize that in tumors the normal signals that result in healing angiogenesis are over-amplified, resulting in support for tumor growth and metastasis. Specifically, we hypothesize that the tumor cells themselves are capable of producing angiogenic factors. In order to test this hypothesis, we examined breast cancer cells by immunohistochemical methods, by homogenization of breast cancer tissue, and by direct measurements from breast cancer cells grown *in vitro.*

Our studies have demonstrated that AF are present in breast cancer tissues. The relationship between the presence of the AF and degree of angiogenesis is being completed. Further we will show that in breast cancer tissue homogenates, the levels of at least one AF, IL-8, correlates with another known predictor of poor outcome in breast cancer, the presence of estrogen and progesterone receptors (ER/PR).

Finally, our *in vitro* studies demonstrated that breast cancer cells are capable of producing AF. Interestingly, this expression is under the control of cytokines normally associated with wound healing such as Interleukin 1 (IL-1).

Body

Specific Aim I- To characterize IL-8 expression in human breast cancer

The majority of the Studies for Specific Aim I were completed during Year 01, and were previously reported in the first Annual Report. During Year 02, we began our analysis of this data, and added several studies that we felt would better characterize the expression of angiogenic factors in tissue from patients with breast cancer.

Study IA- To characterize IL-8 antigen distribution in human breast biopsy specimens

Introduction and Rationale

Angiogenesis is important in the growth and metastases of human breast cancer. We hypothesize that this process is under the control of angiogenic factors such as IL-8. These angiogenic factors are produced either by the tumor cells themselves, or by infiltrating leukocytes. Study IA was designed to demonstrate the presence and distribution of IL-8 antigen in breast cancer specimens. The subsequent studies will determine the source of the angiogenic factors.

Results

In preliminary studies, we found that IL-8 antigen was present in specimens from human breast cancer. This work was extended to include a total of 66 cancers, 10 non invasive cancers and 11 benign tissue specimens. The majority of this work was completed in Year 01 of this project. The materials and methods and results of this study are presented in Abstract 1, and Manuscript 1 included in the Appendix. Briefly, our results confirmed that IL-8 antigen is found in human breast tissue (see Table 1). We were not surprised to find that IL-8 antigen was found in non malignant breast tissue, and non invasive cancer

specimens. IL-8 is a normal component of wound healing and repair. We believe that it is the over-expression of IL-8 that is associated with abnormal angiogenesis. Importantly, the pattern of staining seen in the benign tissues differed from that seen in the malignant tissues. The staining in the benign breast was described as "apical" as compared to the homogenous staining seen in the invasive cancer specimens (see Figure 1).

Future Directions:

The demonstration of IL-8 antigen in breast tissue specimens confirms our initial hypothesis that IL-8 is present in human breast cancer. The original scope of this work included only IL-8. Further, we have begun investigating the presence of IL-8 receptors in the tissue specimens. In addition, further work is needed to identify both the source of the IL-8 as well as the site of action. In order to accomplish this, we have completed our *in vitro* studies (see below). Additionally, in the tissue homogenate work described below, as well as in our *in vitro* studies, we have found that other angiogenic factors such as bFGF, and VEGF also have a role in breast cancer. We have begun examining the tissue specimens by immunohistochemical methods for these other angiogenic factors.

Study IB- To demonstrate IL-8 mRNA expression in human breast cancer tissues

Introduction and Rationale

The purpose of this Specific Aim was to identify the breast cancer cells as the source of the IL-8. However, based on data obtained from the in vitro studies outlined below, we felt that it was no longer necessary to pursue the experiments outlined in Study IB. The results of the in vitro studies clearly demonstrate that beast cancer cells are capable of producing angiogenic factors such as IL-8. Further, we found that this expression was under the control of cytokines.

Future Directions

Based on the other studies presented, Study IB will not be performed. Specific Aim II data will confirm that breast cancer cells are the source of IL-8. This has the advantage of allowing us to study the regulation of IL-8 expression in our *in vitro* model.

Study IC- To Correlate IL-8 expression with neovascularization in human breast biopsy specimens

Introduction and Rationale

It has been clearly demonstrated that angiogenesis is an important factor in predicting outcome in patients with breast cancer. The purpose of Study IC was to correlate the level of angiogenesis as measured by microvessel density with IL-8 antigen expression, as measured by immunohistochemical means. It was anticipated that those tumors which expressed high levels of IL-8 antigen would also have high levels of microvessel counts. The results of this study are presented in Experiment Ic1 below.

In order to further investigate the association between IL-8 expression and outcome, we added an experiment in which we measured the expression of IL-8 in breast tumor homogenates from patients undergoing surgery for breast cancer. The results of the IL-8 levels were then correlated with another known predictor of outcome in breast cancer, estrogen and progesterone receptor status (ER/PR). The preliminary results of this work are presented in Abstract 2, and Manuscript 2. Subsequent to the presentation of this data and the completion of the manuscript, we have extended this work, and the results are presented below.

Experiment Ic1: Microvessel (MVD) correlation with IL-8 staining

Experimental Approach:

Tissue specimens from patients who underwent surgical procedures at the John Dempsey Hospital and Saint Francis Medical Center for benign and malignant breast disorders were identified by searching the Department of Pathology database. Tissue blocks were obtained, and 8μ sections were prepared. These sections were used for the immunohistochemical described studies below and for IL-8 staining in Study IA. Portions of tumors from patients with histologically confirmed invasive breast cancer were sent for ER/PR analysis (see below). These were the samples used for IL-8 level determination (see below).

Immunohistochemical Analysis

Microvessels were identified by immunohistochemical methods. The staining technique was basically the same as outlined in the IL-8 immunohistochemistry section (see Manuscript 1). We chose to use antibody to CD-31 as a marker for blood vessels. We fell that this a more specific agent than anti vWF antibodies. This experience is confirmed by others [7, 8]. Microvessels were identified by the presence of color change of the substrate indicator. This is an accepted method as it includes identification of vessels that are collapsed in which no blood cells can be seen. The technique of counting was identical to methods in published literature. The most prominent area of vessel staining, and the number of vessels in a 200X field were identified using an ocular. The counting was doe by two observers who agreed on the identification of the vessels.

Results

The immunohistochemistry staining, and the vessel counting have been completed. Both staining techniques were successful. To date we have completed CD-31 staining on 47 specimens. Vessel counting has been completed on 19 specimens (see Table 2). No correlation between IL-8 staining and MVD counts was observed either for invasive cancer or for other breast diseases. There are several possible explanations for this result. This could simply be a result of inadequate sampling. We have completed CD-31 staining, and await the results of vessel counting on these specimens. Another explanation is that the subjective evaluation of staining intensity is not an accurate reflection of IL-8 expression in the specimens. Finally, results in other parts of this study have clearly demonstrated that multiple angiogenic factors are present in human breast cancer. Thus multiple angiogenic factors are likely influencing MVD. Correlation with any particular AF may not be useful.

Study IC2: Angiogenic factor levels in breast tissue homogenates

Experimental Approach:

Portions of breast cancer tissue taken form patients undergoing surgery for breast cancer were sent for estrogen and progesterone receptor analysis (ER/PR). Briefly, specimens were immediately shipped to a -70^0 freezer. Before being frozen, they were trimmed of fat, necrotic tissue and normal tissue. He samples were then homogenized in 2.5 ml of buffer under cold conditions, and spun in the ultracentrifuge. Resulting cytosol preparations were transferred to glass tubes. Protein levels were measured using 280Mμ absorption. Samples were then diluted until the protein level was between 1.5-5 mg/ml. The samples were then assayed by standard ELISA methods for ER/PR.

IL-8 levels in the samples were measured using the RIA method developed by us. The method is included in Manuscript 2. The composition of the tissue used for ER/PR and AF analysis varied depending on the nature of the breast cancer. For this reason the AF and ER/PR levels are corrected for the amount of protein in the specimens and expressed as pg. IL-8/mg protein.

Results

The preliminary results of this experiment are presented in Abstract 2 and Manuscript 2. This work was presented at the Forum on Surgical Problems at the Annual Meeting of the American College of Surgeons, October 1996, San Francisco, CA. Subsequent to the preparation of the meeting abstract, we completed IL-8 level evaluation on 111 specimens. In addition, levels of two other known angiogenic factors, vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) were also determined. Elevated levels of all three AF were found in tissue homogenates and are expressed as pg./mg protein (see Figures 2-7).

We examined the correlation between the levels of AF expression among the various AF (Table 3). We found that there was a direct correlation between the levels of IL-8 and VEGF. There was no correlation between IL-8 and bFGF or between VEGF and bFGF. Next, the relationship between AF expression and ER/PR levels was examined (Table 4). Interestingly, we found that the levels of IL-8 were inversely correlated with ER/PR levels. There was no correlation between VEGF levels and ER/PR. Further, there was a direct correlation between bFGF levels and ER which was statistically significant, the correlation with PR approached statistical significance.

Future Directions

Our results indicate that a number of known angiogenic factors are present in human breast cancer. We intend to complete the evaluation of VEGF and bFGF levels in the homogenates. In addition, we will measure the levels of AF receptors in the homogenates. Finally, we will obtain more information regarding the histologic and nuclear grade of these tumors. Since these patients all underwent primary operation for their cancer within the past year, follow up data will not be useful in the immediate future.

Interpretation and Summary of Specific Aim I:

The studies of Specific Aim I clearly demonstrate that AF, specifically IL-8, are present in breast tumor tissue. The immunohistologic results confirm the presence of IL-8 in human breast tissue. We have demonstrated that there is more IL-8 antigen in the specimens of breast cancer. The staining pattern suggests that the breast cells are the source of the IL-8. However, it is possible that IL-8 is produced by infiltrating leukocytes, and that we are demonstrating the presence of IL-8 on receptors on the breast cells. Specific Aim II is designed to demonstrate that breast cells are the source of IL-8 and investigates the regulation of this process.

In order to try and better correlate the amount of IL-8 present with outcome, we measured the level of IL-8 in breast cancer homogenates. We were able to demonstrate that IL-8, VEGF and bFGF are found in many breast cancer specimens. Only IL-8 correlated inversely with ER/PR status. Our interpretation of this is that tumors that express high levels of IL-8 are associated with increased angiogenesis and thus a worse prognosis. The ER/PR status is also a marker of tumors with a worse outlook. Estrogen controls the expression of a number of proteins. We speculate that in breast cells,

estrogen down-regulates the expression of IL-8. The absence of ER prevents this down-regulation from occurring. It is interesting to note that VEGF levels did not correlate with ER/PR, and that bFGF showed a borderline positive correlation. We also compared the levels of the various AF among each other. The fact that only IL-8 and VEGF correlated suggests that the AF expression by human breast cancer is heterogeneous. The observation that the relationship to ER/PR status further implies that the control of angiogenesis, and the response to treatment may be variable. The variation in AF response confirms we are not observing a non-specific response. Future studies aimed at blocking specific AF, and perhaps up-regulating ER/PR may have a role in the treatment of patients with breast cancer.

Specific Aim II- To characterize IL-8 expression by breast cancer cell lines in vitro

Study IIA- To characterize the expression of IL-8 antigen by cultured human breast cancer cell lines

Introduction and rationale:

Our initial goal for these studies of Specific Aim II, was to characterize IL-8 expression and it's relationship to breast cancer cell estrogen dependency *in vitro*. Our preliminary studies indicated that high and low IL-8 expressors existed. To achieve these goals, initially we proposed studying IL-8 expression in two cell lines, BT-20 and MCF-7. Because of the availability of additional tumor and non malignant cell lines, we expanded our studies to undertake a systematic evaluation of IL-8 expression in non malignant, estrogen independent and estrogen dependent breast cell lines (Table 5). In addition to studying baseline expression of IL-8 expression, we expanded our studies to include

stimulation of two known IL-8 inducers, LPS and fibrin as positive controls for our studies.

Experimental Approach:

The complete materials and methods are presented in Manuscript 3 in the Appendix. Briefly, breast cells were trypsinized and placed in culture plates in sufficient numbers to produce confluent cultures. They were allowed to adhere overnight. The media was aspirated, and control media, LPS or fibrinogen was added. The fibrinogen was activated with thrombin. At 4, 24, and 48 hours, the supernatant was harvested and the cells lysed with 0.1% Triton-X. The samples were then frozen at -70^0 C until analyzed. IL-8 levels were determined by an RIA developed in our laboratory (see Manuscript 3).

Results:

The results of this study are presented in the Table 6 and Abstract 3 (see Appendix). Briefly, we found that generally breast tumor cell lines produced low levels of IL-8 when compared to the non malignant cell lines. Interestingly, the virally transformed breast cell line, HBL-100 showed the highest baseline level of IL-8 expression.

When these cell lines were stimulated with fibrin, we found that the estrogen independent cell lines were the most responsive to stimulation, when compared to the estrogen dependent cell lines. Further, the HBL-100 cells, which had the highest baseline IL-8 expression, were not stimulated by fibrin. LPS is a non-specific stimulator of cells. We observed a similar pattern, where IL-8 expression was induced in the same cell lines as seen with fibrin. However, only one of the estrogen independent cell lines, MDA-MB-231 cell line were sensitive.

Future Directions:

These studies clearly indicate that there is heterogeneity in IL-8 expression in the cell lines. They suggest that estrogen dependency is involved in the IL-8 response. Thus, if time and resources allow, we will undertake repeating these studies in the presence of estrogen, and estrogen inhibitors. Additionally, we will correlate this data with the *in vivo* experiments in immunodeficient mice scheduled in Years 03 and 04.

Study IIB- To quantify IL-8 expression in cytokine stimulated breast cancer cells

Introduction and Rationale:

In our original proposal we hypothesized that angiogenic factor expression would be regulated by chemical signals known to present in the tumor micro-environment, i.e. cytokines. Since previously, we have demonstrated the presence of IL-1 and tumor necrosis factor (TNF) in the tumor micro-environment[9, 10], we proposed to utilize these in our *in vitro* studies of IL-8 expression. It should be noted that we expanded the scope of our original proposal by investigating four cytokines as opposed to two, and by the addition of 6 additional cell lines. We feel this will provide a more comprehensive evaluation of the role of cytokines in the regulation angiogenic factor expression by human breast tumor cells.

Experimental Approach:

The complete materials and methods are presented in Manuscript 3 in the Appendix. Briefly, the cells were grown and treated as described for the baseline studies of Study IIA. IL-1 and TNF were added as test stimuli. Cell viability was assessed at each time point. In general, we did not observe cell toxicity in the treated cultures. When the cells did not appear viable, the supernatants and lysates were discarded.

Results:

The results of this experiment are presented in Abstracts 4 and Manuscript 3 in the Appendix. Our studies demonstrate that IL-1 α and IL-1β are extremely potent inducers of IL-8 expression in estrogen independent cells, as compared to estrogen independent cells. Additionally, although a similar pattern of IL-8 expression induced by TNF α and TNFβ is seen in these cell lines, the levels of IL-8 expression are significantly lower than those seen in the IL-1 cultures.

Future Directions:

In summary, we have demonstrated that IL-1 is a potent inducer of IL-8 expression by breast cancer cells. Further, this effect appears related to the estrogen dependency of the cells. This is interesting because it correlates with the breast tissue homogenate data in Specific Aim I, i.e. higher IL-8 levels were found in breast cancer homogenates that demonstrated low levels of estrogen receptors. In future studies , if time and resources allow will include:

1. Study cytokine regulation of IL-8 expression in the presence of estrogen and estrogen inhibitors.
2. Expansion to other angiogenic factors (VEGF and bFGF)
3. Study angiogenic factor and cytokine receptors in all above studies
4. IL-1 and TNF analysis of breast cancer homogenates, and correlation with estrogen receptor levels.
5. Characterize IL-8 expression in implanted tumors in immunodeficient mice (Specific Aim III).

6. Immunohistochemical analysis of human breast cancer tissue for IL-1 and TNF.

Study IIC- To quantify IL-8 expression in co-cultures of MCF-7 and BT-20 cells

Introduction and Rationale:

Regulation of angiogenic factor expression in human breast cancer is a complex process that likely involves receptors, receptor antagonists and inhibitors. We have obtained extensive data regarding the regulation of IL-8 expression in breast cancer cells. It is clear that fibrin and cytokines are important in this process. In our original application, we proposed co-culture experiments aimed at characterizing possible inhibitors. Since our original proposal, we have added 6 new cell lines, and 4 new cytokines. This work has verified the important role of cytokines and fibrin in angiogenic factors, and led us to focus this experiment on IL-8 expression in stimulated conditions. Thus this portion of the experiment was postponed to Year 03.

Conclusions

The studies completed in Years 01 and 02 have clearly demonstrated that IL-8 antigen is found in human breast cancer, and that breast cancer cells are capable of producing IL-8. This work supports our original hypothesis that in the tumor micro-environment, angiogenic factors are produced, and result in increased tumor growth and metastasis. The finding of the regulation of angiogenic factor expression by fibrin, and cytokines associated with wound healing are consistent with the theory that cancers behave like wounds that are healing in an abnormal fashion.

The immunohistochemical, homogenate and *in vitro* studies presented will be key

in supporting the *in vivo* experiments outlined for Years 03 and 04. In these studies, we will first refine our *in vivo* model in immunodeficient mice, and then establish baseline conditions as described for the experiments already completed. Once these baseline conditions are determined, we will attempt to block angiogenesis and tumor growth using specific antibodies. However, based on observations made in Years 01 and 02, we will broaden our approach to include study of the role of estrogens and estrogen antagonists (e.g. Tamoxifen). If time and resources allow, we will also expand to other angiogenic factors such as VEGF and bFGF.

These experiments support the role of anti-angiogenesis treatment in human breast cancer. Based on our observations, multiple angiogenic factors are likely to play a role in this process. It may become important to determine which angiogenic factors are active in any particular patient.

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Statement of Work

Task 1 Year 1- To characterize IL-8 expression in human breast cancer

Study IA- To characterize IL-8 antigen distribution in human breast biopsy specimens

Study IB- To demonstrate IL-8 mRNA expression in human breast cancer tissues

Study IC- To Correlate IL-8 expression with neovascularization in human breast biopsy specimens

Task 2 Year 2- To characterize IL-8 expression by breast cancer cell lines in vitro

Study A- To characterize the expression of IL-8 antigen by cultured human breast cancer cell lines

Study B- To quantify IL-8 expression in cytokine stimulated breast cancer cells

Study C- To quantify IL-8 expression in co cultures of MCF-7 and BT-20 cells

Task 3. Years 3-4- To characterize IL-8 antigen expression and neovascularization in human breast cancer cells grown as tumors in nude mice

Study A- To demonstrate IL-8 antigen expression in subcutaneously implanted human breast cancers

Study B- To establish a dual tumor model using MCF-7 and BT-20 cells

This Statement of Work has been modified to reflect 75% of the original budget. The final study has been omitted due to the lack of resources. If insufficient funds are available, the *in vivo* portions of the project will be further modified upon notification.

Figure 1

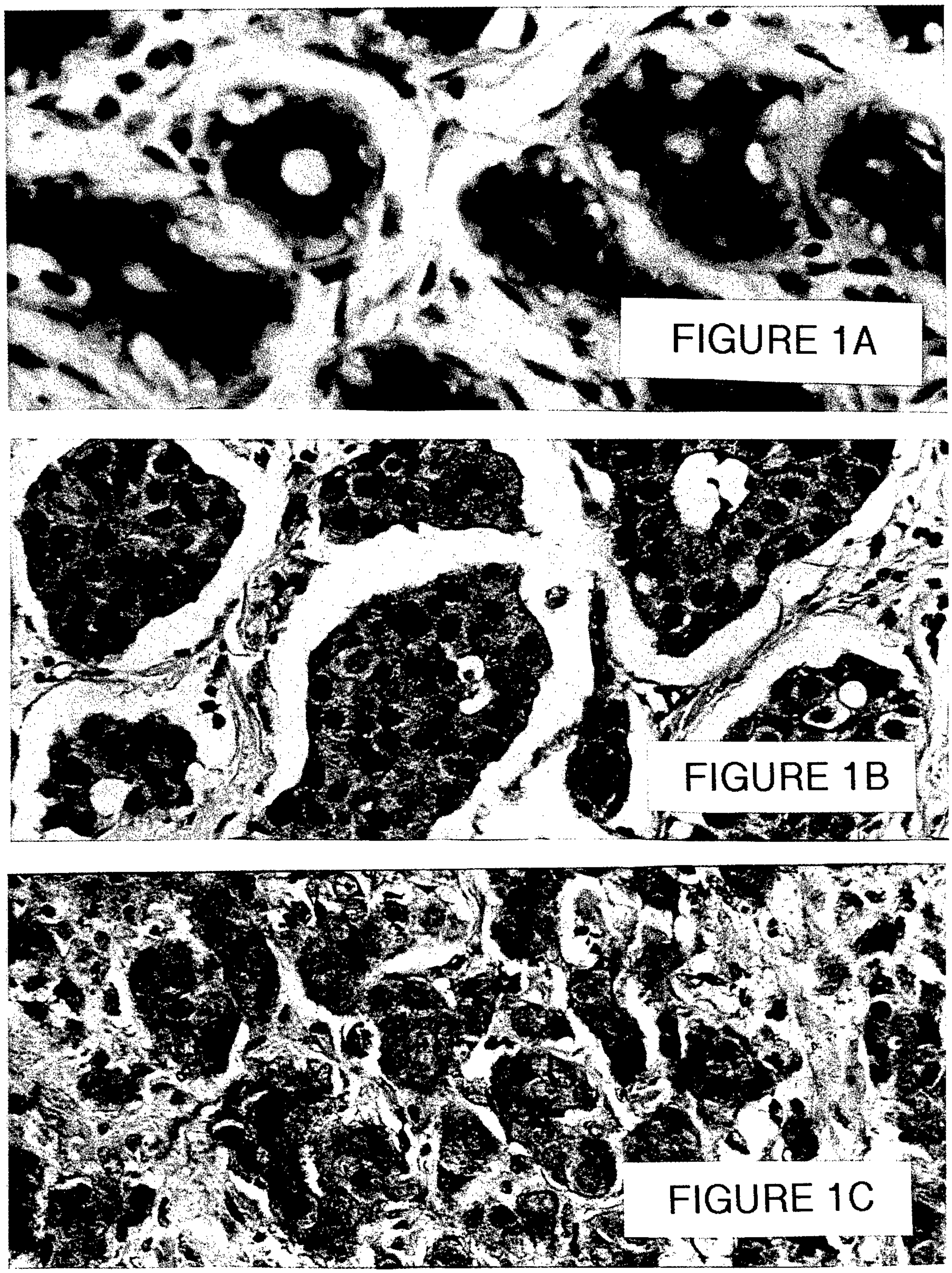


Figure 1: IL-8 staining of A-benign breast tissue, B-*in situ* cancer and C- invasive breast cancer. Note "apical" staining in benign breast, compared to homogenous staining in invasive cancer.

Figure 2

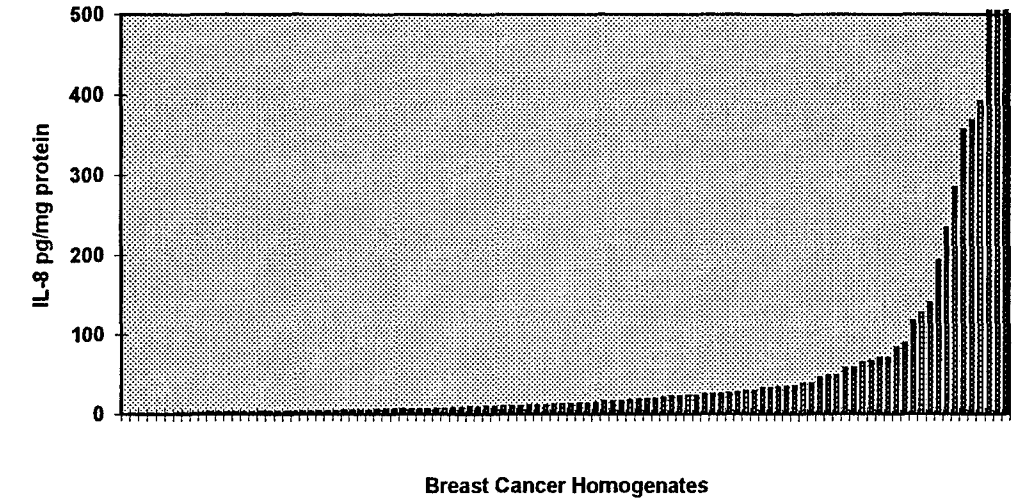
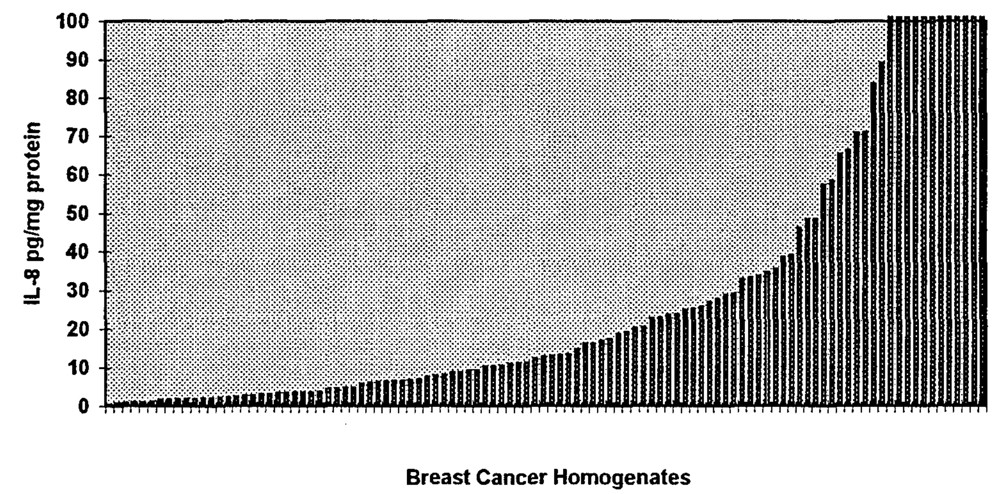


Figure 3



Figures 2 and 3: IL-8 in pg/mg protein in breast cancer homogenates. Note Y axis cut off at 100 pg/mg protein in Figure 3 to illustrate IL-8 levels in low samples.

Figure 4

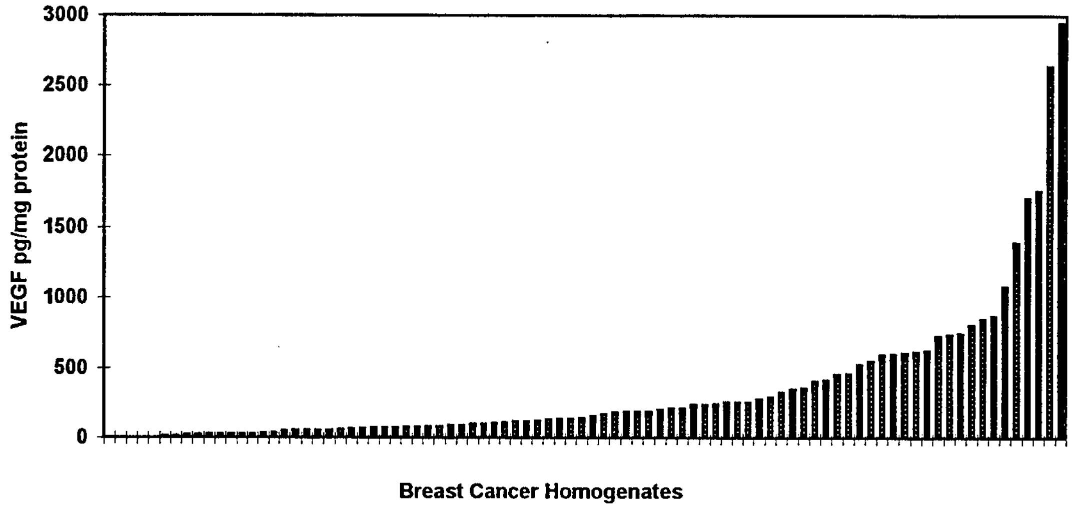
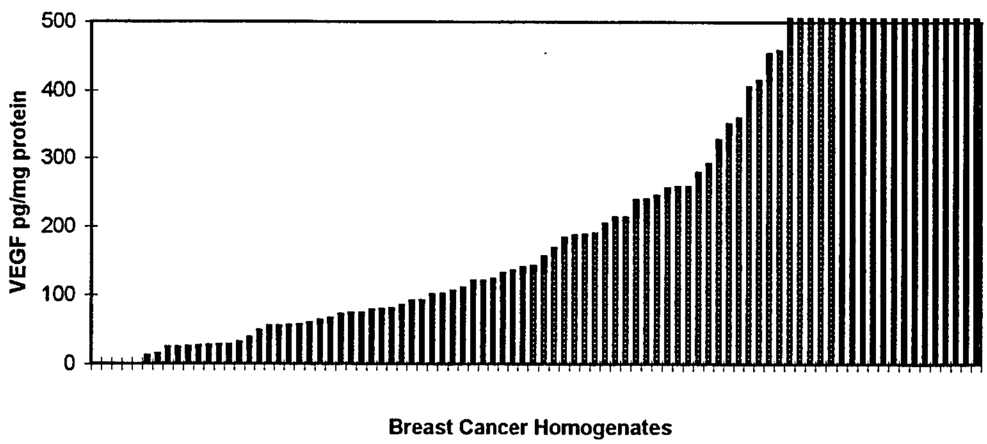


Figure 5



Figures 4 and 5: VEGF levels in pg/mg protein in breast cancer homogenates. Note Y axis cut off at 500 pg/mg protein in Figure 5 to illustrate VEGF levels in low samples.

Figure 6

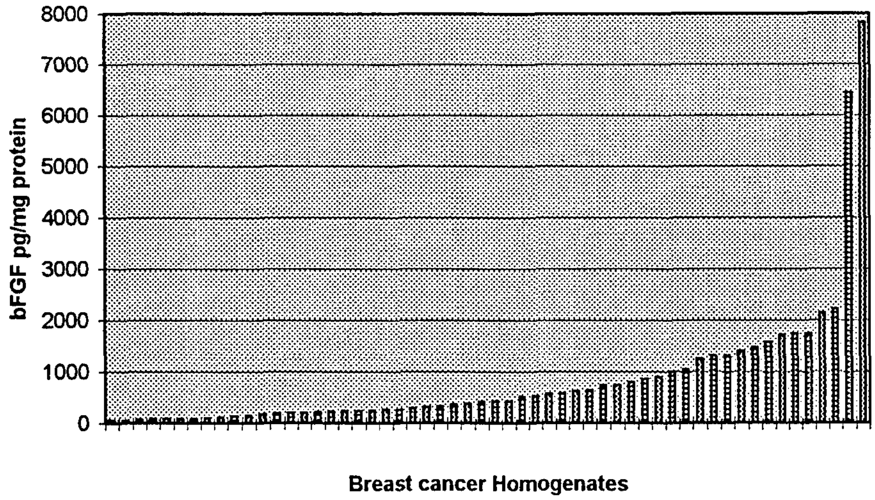
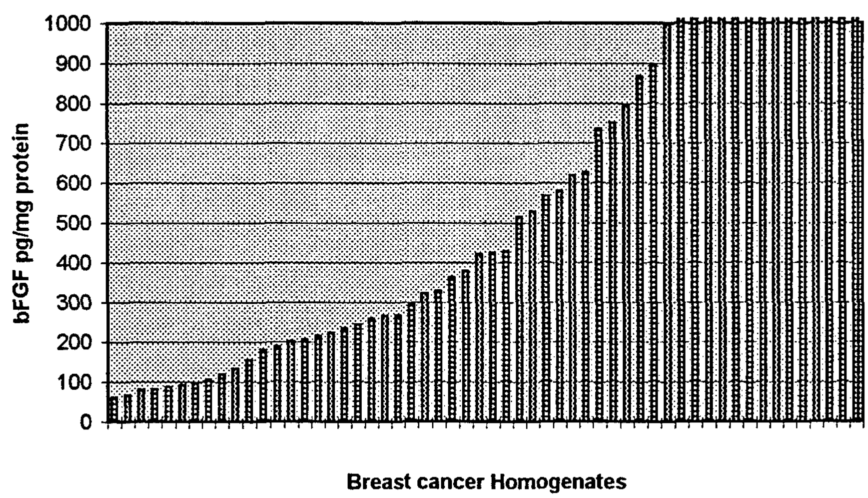


Figure 7



Figures 6 and 7: bFGF in pg/mg protein in breast cancer homogenates. Note Y axis cut off at 1000 pg/mg protein in Figure 5 to illustrate bFGF levels in low samples.

Table 1

Staining	0	1+	2+	3+	4+
Invasive Cancer	1	2	17	11	14
DCIS	1	1	1		
Benign	0	0	3	5	4

IL-8 staining patern seen in invasive cancer, *in situ* cancer, and benign breast tissue. Note that staining was graded as the most intensly staining area. The pattern in the benign tissue was described as "apical" except for the fibroadenomas.

Table 2

Diagnosis	IL-8 staining	Microvessel counts
FA	4	58
FIB	2.5	49
FIB	2	44
FIB	3	38
FIB	3	37
INV	2	76
INV	0.5	46
INV	2	34
INV	2	55
INV	1	41
INV	1	27
INV	2	26
INV	3	56
INV	3	56
INV	4	37
INV	4	41
INV	3	28
INV	2	35
DCIS	2	40

Number of Microvessels per 200X field. Three areas of high vessel numbers were counted. The results shown indicate the highest vessel count of the three areas. (FA=fibroadenoma, INV= invasive cancer, DCIS=ductal carcinoma in situ)

Table 3

	IL-8 vs VEGF	*IL-8 vs bFGF*	*VEGF vs bFGF*
p value	0.0027	n.s.	n.s.

Comparison of angiogenic factors per patient specimen in breast cancer homogenates.

Table 4

	IL-8 (n=111)	VEGF (n=87)	bFGF (n=56)
ER	0.0017 (inv)	0.625	0.0181 (dir)
PR	0.0227 (inv)	0.9215	0.0679 (dir)

Correlation between angiogenic factor level and ER/PR levels in human breast cancer homogenates. The value is expressed as the p value of the correlation. Note inverse corrleation of IL-8 and direct correlation of bFGF.

Table 5

Cell Line	Source
Breast Epithelial Cells	Clonetics
HMEC	ATCC
Hs 578 Bst	ATCC
HBL-100 (SV-40 virally transformed)	ATCC
Estrogen Independent Breast Cancer Cells	
BT-20	ATCC
MDA MB-231	ATCC
Estrogen Dependent Breast Cancer Cells	
MCF-7	ATCC
T-47 D	ATCC
ZR-75-1	ATCC

Cell lines used in *in vitro* studies.

Table 6

Cell Line	Baseline	LPS	Fibrin
Normals			
HMEC	2.30	3.39 (1.48)	4.88 (2.12)
Hs 578 Bst	0.48	7.01 (14.560	7.55 (15.69)
HBL-100	8.80	8.63 (0.98)	3.73 (0.42)
Estrogen-Independent			
BT-20	0.24	0.25 (1.01)	5.61 (22.85)
MDA-MB-231	1.08	136.21 (126.58)	26.67 (24.78)
Estrogen- Dependent			
MCF-7	0.29	0.33 (1.13)	1.21 (4.17)
T-47D	0.11	0.10 (0.89)	0.13 (1.24)
ZR 76	0.24	1.98 (8.12)	0.73 (2.99)

Baseline and fibrin or LPS stimulation of IL-8 expression. Values are exprssed in pg/ml, and the stimulation index (media/stimualtion) is in parantheses.

Abstract 1

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P41)(1994) ANGIOGENESIS, CYTOKINES AND BREAST CANCER: INTERLEUKIN-8 LOCALIZATION IN INFILTRATING DUCTAL BREAST CANCER

---------------------- Author ----------------------

S. Kurtzman, J. Contrino, Y. Ai, M. Sanders, D. Kreutzer. U of Connecticut Health Center, Farmington, CT

---------------------- Body ----------------------

Breast cancers are highly vascularized tumors. Previously, the extent of vascularization (angiogenesis) in breast cancer has been shown to correlate with poor outcome. Interleukin-8 (IL-8) is a recently described cytokine which has been implicated as a potential neovascularization factor in wound healing. While the role of angiogenic factors such as TGF-beta has been described in breast cancer, little is known concerning the role of IL-8. We hypothesized that IL-8 is present in breast cancer and plays a role in tumor neovascularization. The goal of this study was to demonstrate and correlate the presence of IL-8 antigen in malignant breast disease. We therefore investigated the expression of IL-8 antigen in benign and malignant breast disorders by immunohistochemistry. For these studies, formalin-fixed, paraffin-embedded specimens of fibrocystic disease, in situ intraductal breast carcinoma, and infiltrating ductal carcinoma were obtained. Eight micron histologic sections were prepared and analyzed by immunohistochemistry using chicken-anti human IL-8 antibody and the Avidin-Biotin Complex (ABC) staining system. Our studies demonstrated that infiltrating ductal cancer specimens consistently expressed high levels of IL-8 antigen in association with the tumor. In addition, blood vessels in the region of the tumors also expressed high levels of IL-8 antigen. To a lesser extent, IL-8 antigen was expressed in the in situ cancer as well as the fibrocystic tissue, but the intensity was less, and the pattern was variable in both the involved areas and associated blood vessels. These observations demonstrate that a significant correlation exists between IL-8 expression and malignancy in human breast cancer, supporting our hypothesis that IL-8 is involved in neovascularization and perhaps metastasis of malignant breast tumors. Future studies will be aimed at clarifying the exact role of IL-8 neovascularization and tumorigenesis in breast cancer.

Source: Society of Surgical Oncology, Annual Cancer Symposia.

Presented at Annual SSO Symposium, Houston,TX; March 1994

Abstract 2

Surgical Oncology and Related Immunology

INCREASED ANGIOGENESIS FACTOR EXPRESSION IS ASSOCIATED WITH NEGATIVE HORMONE RECEPTOR STATUS IN HUMAN BREAST CANCER

In many ways, tumors share features with wounds including the need for neovascularization. In fact, tumors will not grow larger than a few millimeters unless they are able to recruit new blood vessels. Previous studies have correlated high levels of tumor neovascularization with poor outcome in breast cancer patients. We hypothesize that tumors contribute to their own growth and metastasis by producing angiogeneic factors (AF) such as Interleukin 8 (IL-8). Since breast tumors that exhibit negative hormone receptor status are also associated with poor outcome, we correlated the levels of IL-8 with estrogen and progesterone receptor levels (ER/PR) in breast cancer homogenates from 39 patients. Standard ELISA and RIA methods were used to determine ER/PR and IL-8 levels. Protein levels were determined using the Bradford reagent, data is corrected to mg cytosol protein. ER/PR levels less than 10 fg/mg cytosol protein were considered negative.

IL-8 Responders: ng/mg protein (range)	ER Negative/total	PR Negative/total
Low < 10 (1.9-10)	2/15 (13%)	4/15 (27%)
High >10 (11.2-8276)	12/24 (50%)	15/24 (62.5%)

We found that: 1) there were two populations of breast cancers, low IL-8 responders (n=15) and high IL-8 responders (n=24) and 2) high levels of IL-8 expression were associated with negative hormone receptor status. This data suggests that the poor outcome associated with negative hormone receptor status may be related to increased levels of AF produced by the tumors, which results in tumor growth and metastasis. Further studies to elucidate the interrelationship between AF expression, hormone receptors and poor outcome are in progress.

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Presented at The Surgical Forum, American College of Surgeons Annual Meeting, San Francisco, CA; October 1996

Abstract 3

Fibrin regulation of angiogenic factor expression by human breast cancer cells *in vitro*. Scott H. Kurtzman, Lauri Miller, Kathleen Anderson, Yanping Wang, Donald L Kreutzer; Univ of CT School of Medicine; Farmington CT 06030

Increasing evidence supports a close relationship between fibrin formation in the tumor microenvironment (TME) and tumor angiogenesis. Recent studies in our laboratory have suggested that fibrin is a dynamic, pluripotent activator of a variety of cells in the TME including tumor cells. These observations have led us to hypothesize that fibrin controls angiogenesis in breast cancer by regulating tumor cell expression of key angiogenic factors (AF) e.g. Interleukin 8 (IL-8). In order to test this hypothesis, 7 breast cell lines grown in continuous culture were exposed to fibrin, TNF-α or control media. Culture supernatants were harvested at 24 hours, and assayed for IL-8 by RIA. The ratios of stimulated over control IL-8 levels for the 3 estrogen independent breast cancer cell lines (MDA-231, BT-20, HS-578) were 25, 36 and 9:1 for fibrin stimulation and 12, 2 and 10:1 for TNF-α stimulation. For the 3 estrogen dependent breast cancer cell lines (MCF-7, ZR-75, T-47D) and the 1 normal breast cell line (HBL-100), the IL-8:control ratios were 2, 1, 1 and 0.3:1 for fibrin stimulation and 1, 1, 1 and 3:1 for TNF-α stimulation. We conclude that 1) fibrin is a potent inducer of AF expression in human breast cancer cells *in vitro*, 2) there is tumor cell heterogeneity in this IL-8/fibrin response, and 3) estrogen independent tumor cells produce more IL-8 in response to fibrin than do estrogen dependent cells. Clearly, further studies investigating the molecular basis of the role of estrogen in fibrin induced AF expression in human breast cancer should be carried out.

Presented at the Annual Meeting of the Association for the Advancement of Cancer Research, Washington, D.C.; April 1996

Abstract 4

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112)(1996) CYTOKINE REGULATION OF ANGIOGENESIS FACTOR EXPRESSION IN HUMAN BREAST CANCER.

---------------------- Author ----------------------

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---------------------- Body ----------------------

Currently, little is known about the regulation of angiogenic factor (AF) expression in breast tumor cells. We hypothesize that in breast cancer 1) breast cancer cells produce AF, and 2) this expression is regulated by cytokines. In order to investigate this, 7 human breast cell lines were grown in continuous culture and exposed to cytokines IL-1/alpha, IL-1/beta, TNF/alpha, TNF/beta or control media. Resulting cell culture supernatants were harvested and assayed by RIA for Interleukin 8 (IL-8), a known AF. The results are presented in Table 1 as the ratio of IL-8 levels in stimulated over control condition.

Table 1	BT-20	MDA-231	ZR-75-1	HS-578	T-47D	MCF-7	HBL-100
Media	1.00	1.00	1.00	1.00	1.00	1.00	1.00
TNF α	1.84	12.2	0.73	9.80	0.91	1.30	0.32
TNF β	1.06	1.59	0.68	4.10	0.67	1.00	0.09
IL-1 α	1362	127	107	124	3.36	0.95	0.12
IL-1 β	1292	144	137	119	1.00	0.81	0.24

These data clearly indicate that 1) IL-1 appears to be an extremely potent and consistent inducer of IL-8 in a number of breast cancer cell lines and 2) different tumor cell lines respond differently to individual cytokines (high vs. low responders.) Thus, human breast cancer cells are capable of producing AF in response to cytokines such as IL-1 and TNF. Since IL-1 and TNF are produced within the tumor microenvironment by tissue cells and leukocytes, they likely contribute to angiogenesis and resulting tumor growth and metastasis. This information will allow us to design future therapeutic agents targeted at AF expression and their receptors that will aid in the treatment of patients with breast cancer.

Source: Society of Surgical Oncology, Annual Cancer Symposia.

================== End of abstract ==================

Presented at Annual SSO Symposium, Atlanta, GA; March 1996

DOD Annual report: Manuscript 1

Interlueukin 8 Expression in Human Breast Cancer

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MANUSCRIPT IN PREPARATION; UNPUBLISHED INFORMATION!!!

Abstract

Cancers rely on angiogenesis to support growth and metastasis. We and others hypothesize that tumors influence their own growth in a manner similar to healing wounds. Accordingly, we theorize that tumor cells control their microenvironment by producing cytokines that act in an autocrine and paracrine fashion to stimulate angiogenesis. The cytokine Interleukin 8 (IL-8) has been shown to be a potent mediator of lymphocyte recruitment and neovascularization in the inflammatory response, and is a known angiogenic factor. This study was aimed at determining whether or not IL-8 is expressed in human breast cancers. Breast cancer homogenates were assayed, and elevated of IL-8 were found in the majority of specimens. IL-8 was localized to the breast cancer cells using standard immunohistochemical methods. We examined 45 invasive breast cancers, 3 *in situ* cancers, and 12 normal tissues. We found strong and consistent staining of human breast cancers for the IL-8 antigen in the invasive cancers, moderate and inconsistent staining in *in situ* cancers, and apical staining only in the benign cases. We conclude that IL-8, a known angiogenic factor, is found in human breast cancer. This finiding supports our hypothesis that breast cancer cells are capable of producing angiogenic factors, and thereby support their own growth and metastasis.

Introduction

Breast cancer is the most common malignancy in women in the United States. The overall survival for all patients with this diagnosis is approximately 70%. Factors associated with poor prognosis include presentation with advanced stage disease or invasion of neural and vascular structures. Recently, it has become clear that patients whose tumors demonstrate a high degree of angiogenesis have a shorter disease free survival, and that these patients are more likely to develop distant metastases [Weidner, 1991 #34]. It is also well accepted that tumor can not grow larger than several millimeters in diameter (approximately 10^6 cells) without ingrowth of additional blood vessels [Weidner, 1992 #28]. Angiogenesis has been shown to be critical for the growth and metastasis of human breast cancer [Gasparini, 1995 #43]. Understanding the underlying mechanisms and factors that control angiogenesis will provide not only insights into the basic mechanisms of tumor growth and metastasis, but also will likely provide novel new therapeutic approaches to invasive breast cancer.

Although the relationship between neovascularization and poor outcome in breast cancer is well established, the mechanism of this relationship is not well understood. Undoubtedly, multiple angiogenic factors (AF) are involved in the recruitment and development of tumor angiogenesis [Folkman, 1987 #44]. The possible sources of these factors include the tumor cells themselves, tissue cells or infiltrating leukocytes. Previous studies have demonstrated that AF function by inducing vascular endothelial cell proliferation and/or migration. A variety of AF have been recently identified, and include Interleukin 8 (IL-8), Vascular Endothelial Cell Growth Factor (VEGF), and basic

Fibroblast Growth Factor (bFGF)).

We and others have hypothesized that tumors behave as wounds that do not heal [Dvorak, 1986 #94],[Whalen, 1990 #91]. Normal wounds heal as a result of infiltration of leukocytes and other cells releasing signaling factors which influence the local microenviornment. As a result, a multitude of reactions occur which result in the promotion of wound healing. These reactions include production of fibrin, collagen and other repair factors, as well as an increase in local blood flow to support these activities. This increased blood flow is a result of both local vasodilatation as well as angiogenesis. We hypothesize that in tumors the normal signals that result in healing angiogenesis are over-amplified, resulting in support for tumor growth and metastasis. Specifically, we hypothesize that the tumor cells themselves are capable of producing angiogenic factors. In order to test this hypothesis, we examined breast cancer cells by immunohistochemical methods, by homogenization of breast cancer tissue, and by direct measurements from breast cancer cells grown *in vitro.*

Recently, Interleukin 8 has been shown to be a potent angiogenic factor. In previous work we have demonstrated the presence of IL-8 in head and neck cancer. We therefore hypothesized that IL-8 might also be found in breast cancer and may be acting as an angiogenic factor in this disease.

In order to investigate this hypothesis, we first examined breast cancer homogenates for the presence of IL-8. Once we determined that IL-8 was found in breast cancer specimens, we performed immunohistochemical analyses on paraffin sections from patients with breast cancer to examine where the IL-8 was located. These studies

confirmed that IL-8 was associated with breast cancer cells *in vivo*. In other *in vitro* studies we have investigated the expression of IL-8 by human breast cancer cells. This work has demonstrated that breast cancer cells can be the source of angiogenic factors. This work supports our overall hypothesis, that in the tumor microenvironment, breast cancer cells can produce angiogenic factors, and thus support tumor growth and metastasis.

Materials and Methods

Patient Population

Patients who underwent surgical procedures at the John Dempsey Hospital and Saint Francis Medical Center for benign and malignant breast disorders were identified by searching the Department of Pathology database. The paraffin tissue blocks from these patients were obtained. 8μ sections were prepared and used for the immunohistochemical described studies below.

Institutional Review Board permission was obtained to examine the medical records on these patients. Demographic data such as age, date of birth, age at menarche, age at menopause, family and personal history of breast cancer, use of hormones and oral contraceptives, parity etc. were obtained. Tumor related data such as size, location, TNM stage, and grade were also recorded.

Tissue Homogenates

Portions of tumors from patients with histologically confirmed invasive breast cancer were sent for ER/PR analysis (see below). We obtained the prepared homogenates from 111 of these patients. These were samples were used for IL-8 level determination (see below).

General Histopathology

The cancer specimens were examined by a pathologist at our institution, who classified the lesions benign, *in situ*, or invasive ductal or lobular cancer.

Immunohistochemical Analysis

IL-8 antigen expression was evaluated on tissue obtained from various patients and on mouse tumor tissue (see below). Indirect immunoperoxidase staining of fixed tissue was performed utilizing anti-human IL-8. Biotinylated goat anti-rabbit IgG and biotinylated rabbit anti-goat IgG (Vector, Burlington, CA) were used as secondary antibodies. Tissue was imbedded in paraffin and processed into 4-6 mm sections. Paraffin-imbedded sections were deparaffinized in xylene and rehydrated in graded alcohol (100%, 95%, 75% and 50%). Samples were immersed in methanol containing 0.01 percent hydrogen peroxide (H_2O_2) at 4°C for 15 minutes for inhibition of endogenous peroxidases, allowed to air dry, and then blocked with normal goat serum or normal rabbit serum (Vector, Burlington, CA) at room temperature (RT) for 1 hour. The sections were then washed three times with PBS (pH 7.4) and then primary antibodies, either chicken anti-human IL-8 (1/500 dilution in PBS with 0.5% bovine serum albumin), chicken anti-human IL-8 preabsorbed with recombinant human IL-8, or buffer control, were added and the sections incubated overnight at 4°C.

Following overnight incubation, biotinylated rabbit anti-chicken IgG (Biotin-SP-Affinipure F(ab^1)2 fragment; (Jackson Immunoresearch Labs, Inc., West Grove PA); 1/100 dilution in PBS with 0.5% BSA was applied to the sections and allowed to incubate for one hour at RT. Sections were washed 3X with PBS in between each of the following steps. HRP-streptavidin (Zymed, San Francisco, CA) at a 1:100 dilution in PBS was applied to the sections and incubated at RT for 1 hour. The sections were incubated sequentially with 3-amino-9-ethyl carbazole (AEC) in 0.1 molar sodium acetate buffer (pH 5.0) and 0.03 percent H_2O_2 for 30 minutes at RT. Samples were then counter-stained with Mayer's hematoxylin (Sigma, St. Louis, MO) for 10 minutes, washed

extensively in distilled H_2O, and dipped in dilute ammonium hydroxide. Sections were then mounted in crystal mounting solution (Biomedia, Foster City, CA) and analyzed by light microscopy. Non-immune normal rabbit serum or non-immune normal goat serum served as control anti-sera for these reactions.

The stained sections were reviewed and scored using a standardized data sheet. The histologic diagnosis was confirmed, and the degree of staining was described on a scale of 1 to 4, i.e. no staining, minimal staining, clearly positive, or strongly positive. The pattern of the staining was describes as luminal, inhomogenous or homogenous. In addition, the types of cells staining were recorded.

Cytokine Analysis

Tissue homogenates were evaluated for total protein (TP) content using the Bradford assay technique (Bio-Rad, Richmond, CA). Radioimmunoassays of tumor homogenates and cell culture supernatants were analyzed by an IL-8 specific RIA developed in our laboratory. Briefly, sample (100μl) was incubated at RT for 2 hours with chicken anti-human IL-8 antibody diluted 1:4,000) in RB buffer (1% BSA, 0.1% Triton X-100 in PBS). Chicken antibody to IL-8 was prepared by intramuscular injection of 100μg of recombinant human IL-8 (77 amino acids, Pepro Tech Inc., Rocky Hill, NJ) prepared in *Hunger's* Titer Max (CYTRX Corp., Norcross, GA). Egg yolks containing antibody were processed as previously described (need ref). Antibody titer and specificity were assessed by double-immunodiffusion and immunoelectrophoresis, respectively. Human ^{125}I-IL-8 (NEN Products, Boston, MA) diluted in RB buffer (70-80,000 CPM/ml) was added (100μl) and the reaction mix was incubated overnight (4^0C). Immune complexes were precipitated by adding 100μl affinity purified goat anti-chicken IgG-coupled microspheres (Kirkegaard & Perry Labs Inc., Gaithersburg, MD) diluted in RB buffer (1:20). After incubation (RT, 2 hours), beads were pelleted (2500g for 15 min.), blotted and counted (gamma counter 1

min.). Samples were quantified by reference to a standard curve constructed using rIL-8 standards (0.039-10 ng/ml). Samples were assayed multiple times and results are expressed as the mean $\pm$ S.D.

Standard curves were used to determine the quantity of cytokine in the sample based on the level of radioactivity or spectrophotometric absorbance of each sample using regression analysis. All samples were assayed in duplicate. In the case of the tissue homogenates results are expressed as the number of nanograms of each factor produced per milliliter of homogenate sample, as well as nanograms per milligram of total protein. For ease of analysis, the *in vitro* data are expressed as cytokine index (CI). The CI equals the IL-8 level present in the cytokine stimulated cell supernatants divided by the IL-8 levels in the culture supernatants using control media.

Immunodeficient mouse model

In order to obtain pilot data, an immunodeficient female nude mice was injected subcutaneously with 10^6 tumor cells from a primary breast cancer (gift Fred Rickles). The cells were washed with PBS, and then treated with trypsin. The cells were then centrifuged and resuspended in serum free media at a concentration of 1 X 10^6 cells/ml. The mice will receive a subcutaneous injection of 0.5 cc (5 X 10^5) cells in the lateral thoracic region. The tumor became palpable at approximately 4 weeks. The mouse was sacrificed, and the tumor placed in 10% formalin. Immunohistochemical analysis for human IL-8 was performed.

Eight week old sever combined immunodeficient (SCID) mice were purchased from Jackson Laboratories, Bar Harbor, Maine and maintained by Dr. Dirck Dillehay, Professor of Veterinary Pathology in the Animal Resources Center, Emory University. Four injections of tumor fragments derived from a 59 year old patient with invasive ductal breast carcinoma(obtained from the Cooperative Human Tumor Network, Birmingham

Alabama) were implanted in the flank of a SCID mouse and a tumor nodule harvested 6 weeks later. The tumor sections were fixed in formalin and stained with monoclonal antibodies specific for IL-8 as described below.

Immunohistochemistry was performed on fixed cryostat sections of the tumor. Briefly, formalin fixed samples were blocked with 5% BSA/PBS for 1 hour at room temperature and then incubated for 24 hours at 4^0C with optimal concentrations of mAb (in the presence of 1% BSA), followed by washing (PBS/0.1% BSA) and incubation with biotinylated goat anti-mouse IgG or IgM for 2 hours at room temperature. The samples were again washed with streptavidin-HRP (Bio-Rad) added for 1 hour at room temperature. The slides wee washed and peroxidase activity detected by AEC (0.4% 3-amino-9 ethylcarbazole in N,N-Dimethylformamide) solution.

Statistical Analysis

Statistical analysis of patients included age, past medical history, medications, tumor stage, tumor histology, nodal status, metastases, disease free and overall survival. These parameters were analyzed for clinical significance and correlated with individual cytokine levels and ratios. In these experimental studies, an analysis of variance (ANOVA) was used to determine significant differences among normal, inflammatory control, and cancer patient groups. All analyses were performed with the JMP 3.0 Program (SAS, Cary, NC). A probability of $p < 0.05$ will be considered significant.

Results

Immunohistochemistry

IL-8 antigen was consistently found in specimens from patients with invasive breast cancer. Staining was also found in some cases of *in situ* breast cancer but the pattern was not as strong or homogeneous. Staining of breast epithelial cells was seen in breast tissue specimens as well. The pattern of this staining was distinctly different than that found in malignant breast tissue.

A tumor specimen from a human breast cancer grown in an immunodeficient mouse was also examined. Specific staining of the breast cancer cells with an antibody to IL-8 confirms the existence of IL-8 antigen on human breast cancer cells.

Discussion

Interleukin-8

IL-8 is a member of a family of 8-10 kD cytokines that are involved in pro-inflammatory and reparative processes. Cytokines in this family are basic heparin-binding proteins that display chemotactic activities in vitro and *in vivo* (5). The presence of IL-8 has been extensively described in two inflammatory conditions (i.e. psoriasis and rheumatoid arthritis). These diseases are characterized by exacerbation/remission cycles, host cellular influx and host induced tissue damage.

IL-8 has been shown to be produced by several cell types [Oppenheim, 1991 #57](5) including mononuclear cells, fibroblasts, endothelial cells and keratinocytes. IL-8 antigen, chemotactic activity and mRNA can be induced by IL-1, TNFα and LPS. Regulation of IL-8 production occurs at several levels pre and post-transcriptional that

vary with the cell type analyzed. With IL-1 stimulation, the IL-8 message in monocytes can be detected at one hour, peak by three hour and rapidly decrease [Matsushima, 1988 #66](6). In contrast, the tissue cells, fibroblast and endothelial cells [Sica, 1990 #59](7), treated with IL-1 produce IL-8 mRNA that is detectable by 0.5 hours, peak by two hours and is maintained at high levels up to sixteen hours. The gene for IL-8 is located on human chromosome 4 [Matsushima, 1990 #58](8) and contains sites for functional responsive elements(i.e. promoters) of IL-1 and TNF. This may directly explain the induction of IL-8 mRNA and protein by IL-1 and TNF. IL-8 mRNA can be super-induced with cycloheximide and IL-1 [Mukaida, 1990 #62](9) indicating that regulation occurs at the pretranscriptional level, possibly by repressor protein induction. In a promyelocytic cell line, HL-60 [Kowalski, 1989 #64](10), the post-transcriptional stability of mRNA for IL-8 can be prolonged by adding PMA plus LPS, but neither IL-1 nor TNF was tested. Glucocorticoids (11) and 1,25 (OH)2-vitamin D3 (12) decrease IL-8 mRNA, and the cytokine IL-4 (13) inhibits IL-8 mRNA transcription in macrophages, but not fibroblasts. Therefore, molecular control of IL-8 occurs at all levels of transcription depending upon the cell type analyzed. It should be emphasized that currently, nothing has been published about the production or regulation of IL-8 in cancer in general and breast cancer specifically.

Our results indicate that elevated levels of IL-8 are found in homogenates of breast cancer specimens. However, the source of this angiogenic factor could have been any of the cells found in breast tumors. To identify the location of the IL-8 in the tumors, we performed an immunohistochemical analysis. This clearly demonstrated that the IL-8 was associated with the breast cancer cells, but did not prove that the breast cancer cells were

the source of the IL-8. This observation was corroborated by the positive result in the immunodeficient mouse tumor model. Our next step was to examine the ability of breast cancer cells to produce IL-8. In our *in vitro* studies, we demonstrated that breast cancer cells are capable of producing IL-8 in response to cytokine stimulation. .

Breast cancer relies on angiogenesis to support tumor growth and metastasis. Little is known about those factors that regulate this process. Our central hypothesis is that breast cancer cells promote angiogenesis and thus tumor growth by producing angiogenic factors. This is consistent with our underlying belief that tumors are analogous to abnormally healing wounds. In this model, the breast cancer cells are responding in an aberrant manner to a normal "healing" signal. In normal wound healing, angiogenesis occurs in response to signals such as the release of IL-1 by infiltrating leukocytes. This response is ordinarily self limited. In cancer, however, there is an abnormal outpouring of at least one angiogenic factor, IL-8. The release of this angiogenic factor then supports tumor growth.

Breast cancers are infiltrated by a variety of leukocytes (TIL). These include helper, cytotoxic and suppresser T cells, B cells, Natural Killer cells and macrophages[O'Sullivan, 1994 #87]. T cell infiltration has been associated with both good and poor outcomes [ref could be rosen]. Tumors which are surrounded by a dense leukocyte infiltrate have been associated with increased axillary metastases and a high mitotic frequency [Aaltomaa, 1992 #78] and a negative impact on survival was demonstrated [Elston, 1982 #92](need to check this reference). Tumors with a more benign histology were free of leukocyte infiltration. While such leukocyte infiltration is common, there are few cases where specific TIL recognize tumors and result in tumor

regression. However, these infiltrating cells are capable of producing a variety of cytokines. Direct production of GM-CSF, TNF-α, and IFN-γ have been shown when IL-2 stimulated CD4+ TIL from breast cancers were stimulated with autologous tumor. Non specific production of IL-1β was also demonstrated [Schwartzentruber, 1991 #85]. Vitolo *et al* examined the expression of mRNA for cytokines in TIL found in human breast cancers [Vitolo, 1992 #82]. In that study, mRNA for TNF-α was found in 11/13 specimens, mRNA for IL-2 was uncommon. However, in the 6 cases, where there was a paucity of infiltrating mononuclear cells, rare cells were found which expressed mRNA for TNF-α, and these cells were felt to be in association with angiogenesis which was occurring in the tumor. Some sentence regarding activation of TIL/mucins from this article, role of TIL's in fighting the tumors. Thus, breast tumors are infiltrated by a variety of cells capable of producing cytokines. This cytokine response is stimulated by the presence of tumor cells. The cytokines thereby produced may be responsible for induction of angiogenic factors by the tumor cells themselves. Therefore, tumor infiltration by leukocytes *in vivo* may not only fail to be cytotoxic, but may in fact help support tumor growth and metastasis by facilitating angiogenesis.

The mechanism of this abnormal response is not yet known. While tumor growth and wound healing share some important characteristics, they also differ in several important ways. Wounds and inflammation are characterized by rubor, dolor, and loss of function, all responses to inflammatory cytokines. These responses in one way or another promote a microenviornment where healing can occur. The required steps include angiogenesis which results in nutrients and blood flow needed, and physical protection of the area consequent to pain and immobility. Teleologically, if tumors are to imitate

wounds, the former characteristics are "desirable", but the pain and loss of function might lead to conscious detection of tumors at an early stage. However, we know that most tumors are painless, and asymptomatic until they reach large size.

Several possible explanations exist. One is that the IL-1 receptor is either over-expressed or abnormal on the breast cancer cells. Another is that the gene for IL-8 is abnormal in the breast cancer cells, resulting in over-expression of IL-8 mRNA in the cells.

We have demonstrated that breast cancer cells are capable of producing IL-8, a know angiogenic factor. This was seen both in human tumors as well as in breast cancer cells grown *in vitro*. Interestingly, there was less staining seen in specimens from patients with DCIS. The pattern of staining in normal breast tissue specimens differed from that found in cancer with respect to intensity and location of the IL-8 on the luminal surface. IL-8 is normally expressed in a variety of epithelial cells and is likely a part of the tissue maintenance mechanisms found in normal tissues and organs. It is the over-expression seen in the invasive, and the intermediate expression seen in the non invasive cancer that is the novel observation.

Manuscript 2

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INCREASED ANGIOGENESIS FACTOR EXPRESSION IS ASSOCIATED WITH NEGATIVE HORMONE RECEPTOR STATUS IN HUMAN BREAST CANCER

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ANGIOGENESIS IS CRITICAL for breast tumor growth and metastasis. This process is regulated by a number of chemical signals known as angiogenic factors (AF).[1] One recently identified AF is designated interleukin-8 (IL-8).[2] Although other AF have been implicated in cancer, previously nothing was known regarding the existence and importance of IL-8 in tumor cells in human breast cancer. Recently, we have demonstrated by immunohistochemical methods the presence of IL-8 in the tumor cells found in specimens of human breast cancer.[3]

Previous studies have correlated high levels of tumor angiogenesis with poor outcome in breast cancer patients.[4] Because breast tumors that exhibit negative hormone receptor status are also associated with poor outcome, we hypothesized that the levels of IL-8 in breast tumor tissue homogenates would inversely correlate with estrogen and progesterone receptor levels (ER/PR) in the tissue homogenates. Specifically, specimens with high levels of IL-8 (increased angiogenesis) would be associated with other markers of poor outcome such as lack of ER/PR.

MATERIALS AND METHODS

For these studies, tissues obtained from 39 patients being operated on for breast cancer were sent for ER/PR level determination, which was measured by standard enzyme-linked immunosorbent assay (ELISA). Protein levels were determined in these specimens using the Bradford reagent, and the data are corrected to mg cytosol protein. ER/PR levels greater than 15 fg/mg cytosol protein were considered positive. The tissue homogenates that were prepared for ER/PR analysis were then assayed for IL-8 using standard radioimmunoassay (RIA) technology. The IL-8 levels are expressed in pg/mg cytosol protein. Levels greater than 10 ng/mg cytosol protein were considered positive.

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Correlation of high and low IL-8 responders with ER/PR status in breast cancer homogenates

IL-8 responders: pg/mg protein (range)	ER negative/total	PR negative/total
Low <10 (1.9–10)	3/15 (20%)	5/15 (33.3%)
High >10 (11.2–8,276)	11/24 (47.8%)	16/23 (69.6%)

RESULTS

Of the 39 breast cancer specimens, 14 (36%) were ER negative and 19 (49%) were PR negative. We found that there were two populations of breast cancers, low IL-8 responders (n = 15) and high IL-8 responders (n = 24) (see Table). Of the low IL-8 responders, only 20% were ER negative and only 33.3% were PR negative. Consistent with our hypothesis, the homogenates that had high IL-8 levels (>10 pg/mg cytosol protein) were associated with negative ER (47.8%) and PR (69.6%) status.

DISCUSSION

In order for tumors to grow more than a few millimeters in size, they must induce new blood vessel growth (angiogenesis). This process is regulated by angiogenic factors. In normal wound healing, angiogenic factors are produced by infiltrating leukocytes, endothelial cells, and tissue cells such as fibroblasts. We hypothesize that in the tumor microenvironment, AF are directly produced by tumor cells. This production of AF then supports tumor growth and metastasis in an autocrine manner. As a result, we would expect that tumors that are able to produce AF would be associated with a worse outcome. Because follow-up data were not available on the patients included in this study, we correlated the levels of IL-8 with an established marker of poor patient outcome, lack of ER/PR.

CONCLUSIONS

These data support our hypothesis that the poor outcome associated with negative hormone receptor status may be related to increased levels of IL-8 produced by the tumors. This IL-8 production results in increased angiogenesis, which supports tumor growth and metastasis. Further studies to elucidate the interrelationship between other AF expression (eg, VEGF, bFGF) in breast tumor homogenates, hormone receptors, and poor outcome are in progress. These very interesting initial studies also will need to be validated in a larger patient population and correlated with patient outcome data. In the future,

inhibition of angiogenic factors such as IL-8 or the cytokines that control them may be an important adjunct in the treatment of patients with breast cancer.

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Cytokine Regulation of Angiogenic Factor Expression by Human Breast Cancer Cells *In vitro*

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MANUSCRIPT IN PREPARATION: UNPUBLISHED INFORMATION!!!!!

Abstract

Background: Angiogenesis is critical for breast cancer tumor growth and metastasis. This process is regulated by a number of chemical signals known as angiogenic factors (AF). One recently identified AF is the cytokine Interleukin 8 (IL-8). Although other angiogenic factors have been implicated in cancer, previously nothing was known regarding the existence and importance of IL-8 in tumor cells in human breast cancer. Recently, we have demonstrated the presence of IL-8 in human breast cancer tissue. This locally produced IL-8 can then support angiogenesis and resulting tumor growth and metastasis. Since in inflammatory diseases, IL-8 expression is known to be under the control of cytokines such as Interleukin 1 (IL-1), and Tumor Necrosis Factor (TNF) we hypothesize, that in the tumor microenvironment, IL-8 production is also under the control of these regulatory cytokines. To test this hypothesis, we evaluated the ability of IL-1 and TNF to regulate the expression of IL-8 in human breast epithelial cells (NBEC), and human breast cancer cells (BCC).

Methods: Five human breast cancer cell lines (BCC), and 3 non malignant breast epithelial cell lines (NBEC) were stimulated with IL-1α, IL-1β, TNF-α or TNF-β. The production of Interleukin 8 by the cells was measured. The resulting cell culture supernatants were harvested at 24 hours, and the IL-8 levels in the various culture supernatants were measured by RIA. All data is expressed as pg/ml and Stimulation Index (stimulated/unstimulated IL-8 levels).

Results: Baseline unstimulated IL-8 expression by normal human mammary epithelial

cells, as well as a virally transformed, non malignant breast epithelial cells expressed low levels of IL-8 (0.15-2.29 ng/ml). Baseline IL-8 expression in the two estrogen independent cell lines (BT-20 and MDA-231) and three estrogen dependent cell lines (MCF-7, ZR-75-1 and T 47D) produced similar low levels of IL-8 in non stimulated conditions (0.1-1.5 ng/ml). Analysis of the 24 hour supernatants indicated that there was no stimulation of two BEC s or the estrogen dependent cell lines with TNF-α or TNF-β. Interestingly, TNF-α and TNF-β induced a 3-24 fold increase in one BEC, and a 2-8 fold stimulation in the estrogen independent cell lines BCC. When BEC were stimulated with IL-1, a 5-104 fold stimulation was demonstrated. In the case of the estrogen dependent cell lines, two of the lines (MCF-7 and T-47D) showed little stimulation, while the third, showed 120-134 fold IL-8 levels. The two estrogen independent cell lines showed a 330-1138 fold increase in IL-8 expression when stimulated with IL-1 cytokines.

Conclusion: These observations clearly demonstrate the ability of human breast cancer cells to produce IL-8 *in vitro*. This IL-8 expression can be dramatically upregulated by the cytokine IL-1..

Introduction

General angiogenesis drill and AF

Angiogenesis is critical to tumor growth and metastasis. This process is thought to be controlled by a variety of chemical signals known as angiogenic factors (AF). A number of well established AF such as Vascular Endothelial Growth Factor (VEGF), basic Fibroblast Growth Factor (bFGF), and more recently, Interleukin 8 (IL-8) have been described, and implicated in human cancer. Although the role of IL-8 in other cancers has been reported[von Biberstein, 1995 #155], currently nothing is known about it's importance in breast cancer. To begin to fill this gap in our knowledge, we recently demonstrated the presence of IL-8 antigen in human breast tissue. Using immunohistochemical methods, we found that there was intense IL-8 staining of tumor cells found in samples of human invasive breast cancer specimens. Less intense and inhomogeneous staining was seen in tumor cells in *in situ* breast cancer and only faint, luminal staining of benign breast epithelium in non malignant breast tissue [S. Kurtzman, 1994 #401]. These studies led us to hypothesize, that in the tumor microenvironment, breast cancer cells produce the angiogenic factor, IL-8. We further hypothesize that breast tumor derived IL-8 expression is directly under the control of IL-8 inducing cytokines, such as Interleukin 1 (IL-1) and Tumor Necrosis Factor (TNF) which are likely present in the tumor microenvironment. In order to directly test this hypothesis, we exposed breast cancer cells (BCC), and non malignant breast epithelial cells (NBEC) *in vitro*, to recombinant IL-1 and TNF and measured the induction of AF expression by the breast cells.

The data presented in this study demonstrates not only the ability of human breast cancer cells to produce IL-8, but further that this expression can be regulated by cytokines. Interestingly, IL-1 seems to be an extremely potent inducer of IL-8 expression in estrogen independent breast cell lines. Clearly these *in vitro* studies will provide the foundation for the study of the role of cytokines in the regulation of angiogenesis in human breast cancer, including the use of IL-1 antaonists and inhibitors to suppress IL-8 induced angiogenesis.

Materials and Methods

General reagents

all the cytokines, kits, ria's anything we bought

Primary and continuous cell lines (Table)

Cell Culture

Cell lines were obtained from ATCC or Clonetics, Corp (Table 1). All experiments presented were carried out using early passages of the various cell lines. The cells were initially propagated in T-75 flasks, and then plated in 12 well tissue culture plates 24 hours prior to use. For the purposes of these experiments, the cells used were only tested in conditions that approximated confluent conditions. We determined that 10^6 cells would produce a single monolayer of cells when placed in 12 well tissue culture plates. The exception were the HS578Bst and HMEC cell lines. Due to the large size of these cells, only 5 X 10^5 cells were plated in an effort to avoid a "super-confluent" plate. Previous experience has demonstrated that the cells do not perform properly when they are in this state. The RIA data is not normalized for cell number.

All cells are grown in media containing 10% fetal calf serum (FCS). Estradiol is present in FCS at levels of 26 pg/ml. In addition, phenol red is present in all media. MCF-7 are grown in media containing Minimum Essential Medium (Eagle) with non essential amino acids, L-glutamine and Earle's salts, sodium pyruvate, penicillin/streptomycin (100 U/ml), fungizone and 10% fetal calf serum (FCS). BT-20 cells are grown in media containing Eagle MEM with non-essential amino acids and Earle's salts, L-glutamine, ,

penicillin/streptomycin (100 U/ml), fungizone and 10% FCS. All cells grow as monolayers and are maintained at 37^{0} C in 5% CO_2. They are passaged when confluent using trypsin.

Cytokine stimulation

The NBEC and BCC were allowed to adhere to the plates overnight. Following this, the culture media was aspirated from the plates, and the cells were immediately treated with control media, or media containing the test cytokine IL-1α, IL-1β, TNFα or TNFβ. Control media was the same media used to keep the cells in continuous culture. IL-1 α, and IL-1 β were obtained from R & D Corp (anytown, usa), TNF-α and TNF-β obtained from Pepro Tech (Rocky Hill, NJ). The cytokines were prepared in tissue culture media, and the concentrations used were 10 ng/ml. Cell cultures were exposed for 4, 24 or 48 hours at 37^{0} C. At the end of the exposure period, the cell culture supernatants were aspirated, and frozen at -70^{0} C until analyzed. The adherent cells were treated with 0.1% Triton X in PBS until cell lysis was demonstrated. The cell lysates were then aspirated, and frozen until analyzed.

Immunocytochemistry

IL-8 RIA

NBEC and BCC culture supernatants and lysates were analyzed by an IL-8 specific radioimmunoassay (RIA) developed in our laboratory. Briefly, sample (100μl) was incubated at RT for 1 hour with chicken anti-human IL-8 antibody diluted 1:2,000) in RB buffer (1% BSA in PBS). Chicken antibody to IL-8 was prepared by intramuscular injection of 100μg of recombinant human IL-8 (77 amino acids, Pepro Tech Inc., Rocky

Hill, NJ) prepared in *Hunger's* Titer Max (CYTRX Corp., Norcross, GA). Egg yolks containing antibody were processed as previously described (need ref). Antibody titer and specificity were assessed by double-immunodiffusion and immunoelectrophoresis, respectively. Human ^{125}I-IL-8 (NEN Products, Boston, MA) diluted in RB buffer (70-80,000 CPM/ml) was added (100μl) and the reaction mix was incubated for 1 hour at room temperature. Next, 500μl of fetal bovine serum 10ng/ml) was added as carrier protein. The immune complex was precipitated out of solution using a 40% saturated ammonium sulfate solution, and pelleted (3000 RPM for 20 minutes), blotted and counted (gamma counter 1 min.). Samples were quantified by reference to a standard curve constructed using rIL-8 standards (0.039-10 ng/ml). Samples were assayed multiple times and results are expressed as the mean $\pm$ S.D.

Standard curves were used to determine the quantity of cytokine in the sample based on the level of radioactivity or spectrophotometric absorbance of each sample using regression analysis. All samples were assayed in duplicate. In the case of the tissue homogenates results are expressed as the number of nanograms of each factor produced per milliliter of homogenate sample, as well as picograms per milligram of total protein. For ease of analysis, the *in vitro* data are expressed as cytokine index (CI). The CI equals the IL-8 level present in the cytokine stimulated cell supernatants divided by the IL-8 levels in the culture supernatants using control media.

Data Analysis

blurb on stat analysis TO BE WRITTEN

Results

Cell Culture

Both the NBEC and BCC were found to grow well on tissue culture plates. We found that the cells became adherent and confluent after overnight incubation. Generally, confluent culture wells contain $5X10^5$ - $1X10^6$ cells. Cell viability assessed at each time point and in the presence and absence of cytokine using trypan blue exclusion. No cytoine related toxicity was observed.

Immunocytochemistry

Immunocytochemical analysis of breast cells demonstrated staining of stimulated breast cells (Figure 2). This staining was non uniform

Baseline IL-8 expression

We began our investigation by first determining baseline (non stimulated) expression of IL-8 by breast epithelial cells, i.e. baseline expression of IL-8 by normal BEC, virally transformed BEC, estrogen dependent BCC and estrogen independent BCC. For these baseline studies, IL-8 levels were determined in 24 hour cell culture supernatants and lysates from non cytokine stimulated cells. In general, IL-8 levels found in the cell lysates paralleled the levels seen in the supernatants being approximately 10% of the supernantant level. The only cell line with significant amounts of IL-8 in the lysates was the IL-1α and IL-1β stimulated MDA MB 281 cells, in this line the amount in the lysate was 25-33% that found in the cell culture supernatants. For this reason, the cell lysate data is not presented. The results of the studies are presented in Figure 2 and Table 2 and

discussed below.

Baseline IL-8 Expression: Breast Epithelial Cells:

Baseline IL-8 expression were initially determined in culture supernatants from normal breast cell lines HMEC, and Hs 578 Bst. As indicated in Table 1, HMEC cells produced 2.3 ± 0.43 pg/ml IL-8 as compared to 0.48 ± 0.07 pg/ml for the Hs 578 Bst for the BEC. Parallel analysis of the cell lysates demonstrated low levels of cell associated IL-8 in both normal cell cultures (<1 pg/ml).

Transformed Breast Epithelial Cells

Interestingly, the SV-40 transformed BEC cell line, HBL-100 demonstrated significantly higher baseline IL-8 levels (8.8 ± 1.43 pg/ml) in the cell culture supernatants when compared to either of the normal BEC lines (HMEC 3.83 fold, Hs 578 Bst 18.33). This elevation of the IL-8 levels was also seen in the cell associated lysates (4.7 pg/ml).

Baseline IL-8 Expression: Estrogen Dependent Breast Cancer Cell Lines

After establishing baseline expression of IL-8 in the non malignant breast cell lines, we next examined the baseline levels in three human estrogen dependent breast cancer cell lines, MCF-7, T-47 D, and ZR-75-1. All three cell lines showed extremely low levels of baseline IL-8 expression in the cell culture supernatants (range: 0.1-0.3 pg/ml) and lysates (range: 0.07-0.15 pg/ml). These levels were ½ to $1/10^{th}$ that seen in the normal BEC.

Baseline IL-8 Expression: Estrogen Independent Breast Cancer Cell Lines

Baseline IL-8 levels in cell culture supernatants and lysates were determined in the two estrogen independent human breast cancer cell lines, BT-20 and MDA-MB-231.

Interestingly, much like the estrogen dependent BCC, the BT-20 cell culture supernatants showed low levels of IL-8 expression in the unstimulated condition. The MDA-MB-231 supernatant levels of IL-8 were similar to the normal BEC (1.08 pg/ml). The levels in the cell lysates (need to check this).

TNF stimulation of IL-8 Expression

Both TNF-α- and TNF-β have been found in a variety of human tumors using both immunohistochemical[Pusztai, 1994 #7] and ELISA techniques. In work done in our laboratory, elevated levels of TNF-beta were found to be three times higher in tumor homogenates from patients with head and neck cancer when compared to levels in tissue from non cancer patients[von Biberstein, 1995 #155]. Additionally, previous studies have shown the cytokine TNF to have a variety of anti tumorigenic and pro tumorigenic activities. For example TNF-β has been shown to be tumoricidal in a limited number of tumor cell lines *in vitro*. TNF-α has been found to have a dual role in angiogenesis; low levels of TNF-α stimulate angiogenesis, and high rates inhibit this process [Fajardo, 1992 #160]. This data led us to hypothesize that the pro-tumorigenic activity of TNF was a result of TNF induced AF expression. In order to test whether TNF-α or TNF-β could induce IL-8 expression from breast cells, we measured the levels of IL-8 in 24 hour cell culture supernatants and lysates from NBEC and BCC treated with either TNF-α or TNF-β (see Figure 2 and Table 2).

TNF: Normal Breast Epithelial Cells

In an effort to establish the effects of TNF-alpha and TNF-beta on IL-8 expression in normal cells, we utilized the HMEC, and Hs 578 Bst BEC. In the case of Hs 578 Bst,

TNFα induced a 23.60 fold stimulation of IL-8 expression [stimulation index(SI)=23.60] when compared to unstimulated (media) control supernatants. Interestingly, there was minimal stimulation of IL-8 by TNF-α with the HMEC cells (SI=1.31). A similar pattern of stimulation was seen for TNF-β in cell supernatants and lysates. Thus, these two BEC show a differential response to stimulation by this cytokine. (Talk about significance related to wound healing)

The transformed BEC showed only modest stimulation of IL-8 expression by TNF-α (SI=2.05) or TNF-β (SI=1.35). (chek lit, talk about viral infections causing derangement and effect on TNF responsiveness)

TNF: Estrogen Dependent Breast Cancer Cell Lines

We next tested the ability of TNF to induce IL-8 expression in the estrogen dependent BCC. As can be seen in Tables 1 and 2, neither TNF-α or TNF-β stimulation resulted in induction of IL-8 by any of the estrogen dependent cell lines. In fact in the case of the ZR-75-1 BCC there was modest inhibition of IL-8 expression. This was not a result of cell toxicity or loss as judged by trypan blue exclusion and cell counting. Check this with Lauri/literature. Thus neither TNF-α or TNF-β appear to be inducers of IL-8 expression by estrogen dependent BCC.

TNF: Estrogen Independent Breast Cancer Cell Lines

In parallel studies, we examined the ability of TNF-α and TNF-β to induce IL-8 expression in estrogen independent BCC. We found that there was modest induction of IL-8 in the MDA-MB-231 BCCs by TNF-α (SI=6.78) and TNF-β (SI=1.89). In contrast, there was no IL-8 stimulation seen in the BT-20 cells in response to either TNF-α or

TNF-β.

These data indicate that there is a heterogeneous of breast cells to TNF stimulation. This phenomenon likely reflects the pattern seen in normal breast tissue and in patients with malignancies. (Mention receptors in the discussion section)

IL-1 stimulation of IL-8 Expression

IL-1 cytokines are known to be a potent inducers of IL-8 expression in leukocytes and normal tissue cells. Therefore, we extended our studies to investigate the ability of IL-1-alpha and IL-1 beta to induce IL-8 expression in normal and malignant breast epithelial cells.

IL-1: Breast Epithelial Cells

Both normal BEC showed marked increased expression of IL-8 when treated with either IL-1 alpha or IL-1 beta (Tables 1 and 2). There was nearly a 10 fold difference in the responsiveness of the two cell lines (HMEC SI=10.91, Hs 578 Bst SI=80.03) in response to IL-1 alpha, as well as in response to IL-1 beta (HMEC SI=6.56, Hs 578 Bst SI=103.81). IL-1 alpha and IL-1 beta produced greater stimulation than either TNF-alpha or TNF-beta in both cell lines (we probably should just delete this sentence, we shouldn't talk about TNF here, and there are too many numbers to make a ratio). We also noted that the Hs 578 Bst lines were more responsive to cytokine stimulation as compared to the HMEC. This emphasizes the heterogeneous in responsiveness both between cell lines, as well as in respect to various cytokines. (Make comment about wound healing vis a vis normal cells)

Analysis of the of induction of IL-8 expression in SV-40 transformed BEC line

HBL-100 in response to IL-1 alpha or IL-1 beta IL-1 stimulation demonstrated that both IL-1 alpha and IL-1 beta induced high levels of IL-8 expression (IL-1 alpha SI=6.88, IL-1 beta SI=5.24) in these cells. This shows that the response to IL-1 cytokines is consistently seen in non malignant BEC. The response of these cells to IL-1 cytokines, but not to TNF cytokines indicates that the cells are capable of IL-8 induction, and that this response is specific to IL-1.

IL-1: Estrogen Dependent Breast Cancer Cell Lines

Evaluation of the ability of IL-1 alpha or IL-1 beta to induce IL-8 expression in estrogen dependent cell lines demonstrated a clear heterogeneity in cell response. For example, MCF- 7 cells showed no response to stimulation (IL-1 alpha SI=1.08, IL-1 beta SI=1.37). The T-47 D BCC showed intermediate responses to IL-1 stimulation (IL-1 alpha SI=2.89, IL-1 beta SI=3.23), and the ZR-75-1 BCC demonstrated profound stimulation (IL-1 alpha SI=120.26, IL-1 beta SI=133.99). These demonstrate once again the heterogeneity in IL-8 expression in estrogen dependent BCC lines. Further, this data clearly shows that these cells can be induced to produce IL-8 and that this response is specific to IL-1 cytokines.

IL-1: Estrogen Independent Breast Cancer Cell Lines

In order to evaluate the ability of IL-1 cytokines to induce IL-8 expression in estrogen independent BCC, we measured the IL-8 expression in cell culture supernatants and lysates at 24 hours in response to IL-1 alpha or IL-1 beta stimulation. As can be seen in Tables 1 and Table 2, IL-1 cytokines were extremely potent inducers of IL-8 expression in both cell lines. IL-1 alpha and Il-1 beta induced SI of over 300 fold in the MDA-MB-

231 cells, and approximately 1,000 fold in the BT-20 BCC. This data not only emphasizes the differential expression of IL-8 by benign and malignant breast cells in response to various cytokines, but also demonstrates that a marked heterogeneity exists in breast cell responsiveness *in vitro.* this sentence needs to be fixed differnetial to cytokines and cells. In vitro and likely in tumor microenvironemnt in vivo.

Vascular Endothelial Growth Factor(VEGF)

VEGF has previously been demonstrated to be a powerful AF. Thus, we also evaluated the normal, transformed, and malignant BCC cell culture supernatants and lysates using a commercially available RIA kit (source ?R&D). The lower limit of the sensitivity of this assay is xyz pg/ml. We were unable to detect any VEGF in any of the culture supernatants or lysates for any of the cells tested.

Discussion

The transformation from benign breast tissue to a malignant phenotype is undoubtedly a multi-step process. In order to be established, and metastasize, cells must demonstrate uncontrolled growth, the ability to penetrate normal tissue planes, and establish new growth in a separate environment. One of the events required for tumor growth to occur is the need for angiogenesis in order to supply the tumor cells with both oxygen and nutrients. It has been demonstrated that tumors can not grow more than a few millimeters in size unless they can successfully induce new blood vessel formation [Folkman, 1987 #44]check this ref. Neovascularization may also allow tumors to have access to the vascular system thus promoting metastases. This fact has supported by a number of recent articles demonstrating the association between the number of microvessels in human cancers, and the likelihood of metastases.

The ability to recruit new blood vessels is required for tumor growth and development. Recent studies have demonstrated that in *in vivo* models, if angiogenesis is blocked, tumors do not grow or metastasize. The purpose of this study was to demonstrate that breast cancer cells are capable of participating in this process, and are in fact the source of the angiogenic factors that support their own growth. Importantly, the breast cancer cells that we tested were found to be very sensitive to cytokine stimulation. Stimulation of the BCC with IL-1 resulted in production of increased levels of IL-8. Interestingly, the estrogen independent cells were found to be more sensitive to cytokine stimulation than the estrogen dependent cell lines. We feel that this exaggerated response results in the angiogenic ability of breast cancer. While the importance of angiogenesis

and angiogenic factors have been described, little is known about the regulation of angiogenesis in human cancer.

Angiogenesis

In order for tumors to grow more than a few millimeters in size, they must recruit new blood vessel growth. This process, called angiogenesis or has been described in a large variety of human malignancies. In several clinical reports, the degree of angiogenesis has been shown to correlate with poor patient outcome. The development of angiogenesis is under the regulation of molecules called angiogenic factors (AF) [Folkman, 1987 #44]. Interestingly, while the identity of these AF has been described, and the importance of the process well accepted, little is known about the regulation of AF expression by human cancers. It is well accepted that tumor can not grow larger than several millimeters in diameter (approximately 10^6 cells) without ingrowth of additional blood vessels [Weidner, 1992 #28]. Thus, understanding the underlying mechanisms and factors that control angiogenesis will provide not only insights into the basic mechanisms of tumor growth and metastasis, but also will likely provide novel new therapeutic approaches to invasive breast cancer.

Angiogenesis is important in cancer/specifically breast cancer

Recently, it has become clear that patients whose tumors demonstrate a high degree of angiogenesis have a shorter disease free survival, and that these patients are more likely to develop distant metastases [Weidner, 1991 #34]. This phenomenon has now been described in a number of human cancers including breast [Weidner, 1992 #28], lung

[Yamazaki, 1994 #327; Fontanini, 1995 #338; Jaeger, 1995 #339; Macchiarini, 1994 #323], prostate [Weidner, 1993 #320; Fregene, 1993 #324], rectal [Saclarides, 1994 #333], testicular [Olivarez, 1994 #331], bladder [Jaeger, 1995 #339], melanoma [Cockerell, 1994 #345; Barnhill, 1993 #321; Guffey, 1995 #336; Barnhill, 1992 #340], and myeloma [Vacca, 1994 #325; Vacca, 1995 #319].

The influence of angiogenesis on outcome in breast cancer has been examined in several studies. In a review of 211 node negative breast cancer, the number of intra-tumoral vessels was confirmed to be a highly significant factor in relapse free survival and overall survival [Bevilacqua, 1995 #388]. In a study by Fox et al, vascular counts in primary tumors predicted both relapse free survival (RFS) and overall survival (OS) in breast cancer specimens, while in that review, ER receptor status did not [Fox, 1994 #6]. The presence of endothelial growth factor receptor (EGFR) also was associated with a significant reduction in RFS in highly vascularized tumors in that study. A positive relationship between VEGF, a potent angiogenic factor, and microvessel density was demonstrated by Toi *et al* [Toi, 1994 #22; Toi, 1995 #389]. In those studies, a significant relationship was found between microvessel density and RFS in both node negative, and node positive patient. The number of microvessels correlated positively with the number of nodes. Thus angiogenesis has been shown to be an important prognostic feature in invasive breast cancer.

Our Previous work (immunohisto

What is IL-8

IL-8 is a member of a family of 8-10 kD cytokines that have previously been shown to be involved in proinflammatory and reparative processes. Cytokines in this family are basic heparin-binding proteins that display chemotactic activities in vitro and *in vivo* (oppenheim). The presence of IL-8 has been extensively described in two inflammatory conditions (i.e. psoriasis and rheumatoid arthritis) [Nickoloff, 1994 #98; Brennan, 1990 #106; Endo, 1991 #110; Lindley, 1991 #121; Gillitzer, 1991 #112; Koch, 1991 #228; Opdenakker, 1991 #254; Peichl, 1991 #256]. These diseases are characterized by exacerbation/remission cycles, host cellular influx and host induced tissue damage.

IL-8 has been shown to be produced by several cell types [Oppenheim, 1991 #299] including mononuclear cells, fibroblasts, endothelial cells and keratinocytes. IL-8 antigen, chemotactic activity and mRNA can be induced in various cells by IL-1, TNFa and LPS. Regulation of IL-8 production occurs at several levels pre and post-transcriptional that vary with the cell type analyzed. With IL-1 stimulation, the IL-8 message in monocytes can be detected at one hour, peak by three hour and rapidly decrease [Matsushima, 1988 #69]. In contrast, the tissue cells, fiberglass and endothelial cells [Sica, 1990 #59], treated with IL-1 produce IL-8 mRNA that is detectable by 0.5 hours, peak by two hours and is maintained at high levels up to sixteen hours. The gene for IL-8 is located on human chromosome 4 [Mukaida, 1990 #68] and contains sites for functional responsive elements(i.e. promoters) of IL-1 and TNF. This may directly explain the induction of IL-8 mRNA and protein by IL-1 and TNF. IL-8 mRNA can be superinduced with

cycloheximide and IL-1 [Mukaida, 1991 #248](need to check ref) indicating that regulation occurs at the pretranscriptional level, possibly by repressor protein induction. In a promyelocytic cell line, HL-60 [Kowalski, 1989 #64], the post-transcriptional stability of mRNA for IL-8 can be prolonged by adding PMA plus LPS, but neither IL-1 nor TNF was tested. Glucocorticoids [Seitz, 1991 #270]and 1,25 (OH)2-vitamin D3 [Larsen, 1991 #120] (need to check ref) decrease IL-8 mRNA, and the cytokine IL-4 [Standiford, 1990 #67] inhibits IL-8 mRNA transcription in macrophages, but not fibroblasts. Therefore, molecular control of IL-8 occurs at all levels of transcription depending upon the cell type analyzed. **It should be emphasized that currently, nothing has been published about the production or regulation of IL-8 in neovascularization in cancer in general and breast cancer specifically.**

. IL-8 is a normal component of physiological angiogenesis such as that associated with wound healing and organogenesis during fetal development. As such, most epithelial cells are capable of producing angiogenic factors such as IL-8, and baseline expression of IL-8 in immunohistochemical staining of normal breast tissue can be found. In our earlier work using immunohistochemical methods, we showed that highly elevated levels of IL-8 staining in specimens of invasive breast cancer from patients. Lesser degrees of inhomogenous staining was found in specimens from patients with *in* situ breast cancer. When specimens of normal breast tissue were examined, only light staining was found, and this was in a luminal pattern, as compared to the diffuse patter n seen in the specimens from cancer patients.

Restate hypothesis general

Thus, angiogenesis is an important process that occurs in human breast cancer, IL-8 is an established angiogenic factor, and IL-8 is found in breast cancer specimens demonstrated by immunohistochemical methods. Experimental evidence has shown that blocking angiogenic factors will inhibit angiogenesis in *in vivo* models[Hu, 1994 #53]. Understanding the mechanisms that control this process will likely lead to new treatment strategies to prevent the growth and spread of breast tumors.

These observations have led us to hypothesize that in the tumor microenvironment, breast tumors themselves produce AF. These angiogenic factors then act n an autocrine and paracrine fashion and support tumor growth and metastasis. Further we hypothesize that this production of AF is under the control of cytokines such as IL-1 and TNF.

The literature shows that Cytokines regulate IL-8

The ability of cytokines to induce neovascularization in a rat sponge model has been demonstrated. When rats were given IL-1α, IL-8, TNF-α, or bFGF, angiogenesis was induced. This induction was blocked by IL-1 receptor antagonist, IL-8 antiserum, TNF-α antibody or anti bFGF antibody[Hu, 1994 #95]., The neovascularization response was accelerated when IL-1-α and IL-8 were co-administered. Interestingly, this augmented response was not seen when TNF-α and IL-8 were co-administered[Hu, 1993 #55]. These studies suggest that an important regulatory relationship exists between specific cytokines.

The regulation of IL-8 expression in non malignant cells has been studied *in vitro*. For example, the expression of IL-8 mRNA by cultured endothelial cells, dermal fibroblasts,

monocytes and keratinocytes in response to IL-1α has been investigated. In that study, endothelial cells produced high levels of IL-8 mRNA, fibroblasts and monocytes produced intermediate levels, and keratinocytes low amounts [Kristensen, 1991 #118].

IL-1/TNF in cancer/breast cancer (Betsy's paper)

What do we want to say here?

Restate hypothesis specific, IL-8 controled by IL-1 and TNFs

Go over how our data shows this (nl cels model for wound healing)

Significance: General Model (wounds like cancers)

The transformation from a mature, differentiated epithelial cell to a malignant cell, capable of growth and metastasis is a multi-step process. In order to assume a malignant phenotype, the cells must develop the ability to be nourished, invade into the lymphatic or vasculature system, and establish growth at distant sites.

Early on in this process, the cancers behave much like healing wounds (Table 2). Like wounds, the tumors recruit leukocytes, promote fibrin deposition, and establish a blood supply. In the healing wound, the signals for these activities are eventually turned off, and the healing process ends. We and others view cancers as persistent sources of irritation, that perpetuate this healing process, in an abnormal, uncontrolled fashion [Whalen, 1990 #91; Dvorak, 1986 #94]. Further, it is our belief that the tumor cells facilitate this in a paracrine fashion, by overreacting to normal healing signals with the production of, among other things, angiogenic factors such as IL-8. Those tumors that are successful in this process, go on to become clinically apparent cancers, and produce

metastases. We expect, that there are other cells undergo malignant degeneration, but be incapable of production of those factors needed to sustain their growth. These tumors would be judged clinically benign, or might not grow to a size where they could be detected. Their fate then would be to remain unnoticed, or to eventually detected by immune surveillance and be destroyed.

It has been stated that cancers behave like wounds that won't heal [Whalen, 1990 #91; Dvorak, 1986 #94]. Tumors and wounds share many features such as infiltration with leukocytes, fibrin production, and angiogenesis. We feel that at the tissue level, tumors produce a chronic irritation. This irritation leads to an attempt to heal the area. In order to accomplish this, infiltrating leukocytes enter the area, and release mediators such as Interleukin 1 (IL-1). These mediators then begin the healing process by recruiting the cells needed, inducing inflammation, and neovascularization. In healing wounds, this process is self limited. In cancers however, these processes may be over-expressed, and result in support for tumor growth and metastasis. We hypothesize that those malignant cells that are capable of responding to these stimuli by producing angiogenic factors (AF) then go on to form tumors that are clinically apparent. The resulting neovascularization also allow access of the cancer cells to the vascular and lymphatic system and can thus facilitate metastasis. Those cancerous cells that do not respond can not grow, and remain indolent.

Significance cancer/breast cancer

Control of cytokines/af will control cancer growth

A number of clinical trials using anti-angiogenesis agents have been carried out or

are under current studies in cancer and in non cancerous neovascular diseases. Some important anti angiogenesis agents include platelet factor 4, carboxyaminotriazole, BB-94 and BB-2516, metalloproteinase inhibitors, thalidomide, interleukin 12, and linomide [Folkman, 1995 #316]. The inhibition of tumor growth by control of angiogenesis would seem to be an effective and well tolerated approach. Inhibition of angiogenesis is directed at new vessel growth. Unlike chemotherapy, this approach is not cytotoxic to endothelial cells, and is therefore not likely to have major effects on established circulation.

Similarly, anti-angiogenesis agents would not be expected to be effective against established tumors with mature blood supplies. The anti-angiogenesis approach would be used in the adjuvant setting to prevent the emergence of tumor cells from a dormant state. As articulated by Folkman, this approach would be useful in combination with chemotherapy. Resistance to this form of therapy does not seem to be a problem in long term studies carried out thus far.

Conclusions

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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